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Immunoelectron microscopy in kidney research: Some contributions and limitations

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An increasing number of specific molecules, mostly proteins, have been purified from renal glomeruli and tubules in the last few years and have been used as antigens to prepare specific antibodies. In addition, monoclonal antibody libraries are being generated against renal components [1–6]. These antibodies have proved to be valuable tools for immunocytochemical studies because important information has been provided by the precise localization of the original antigens at the light microscopical and ultrastructural (electron microscope) level in kidney tissue in situ. Immunofluorescence and immunoperoxidase techniques evaluated by light microscopy are valuable for demonstrating the region of the kidney or cell types in which the antigen is present, but for specific localization (that is, in components of glomerular capillaries and in specific cell structures) the higher resolving power of immunoelectron microscopy is required. In the last five years, there have been significant improvements in the techniques and reagents available for immunoelectron microscopy which have facilitated their application in nephrology.

In this review, we summarize our experience using immunoelectron microscopy as a major tool in our research on several renal, mostly glomerular, antigens. We have summarized what we have learned using these techniques, and included a short discussion of some technical and conceptual aspects and problems encountered in their application. As examples, we have chosen to present the results obtained on the immunoelectron microscopical localization of gp330, the pathogenic antigen of Heymann nephritis [7] and podocalyxin, the major glomerular epithelial sialoglycoprotein [8], as well as several of the major components of the GBM, heparan sulfate proteoglycans (HSPG) [9], laminin [10], and type IV collagen [10].

The technical and interpretative principles derived from these examples have proved to be applicable to other systems, including antigen localization in various organs [11–15], in cultured cells in vitro [16], and in subcellular fractions [17].

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Strategies for immunoelectron microscopical localization

General comments

The primary problem in immunocytochemistry is to adjust the delicate balance between adequate preservation of morphological structure and maintainance of antigenicity, while assuring accessibility of antibodies to their antigens. In our experience, the conditions of fixation and incubation must be determined and optimized for each antigen.

The methods available for antigen localization by immunoelectron microscopy can be classified as either "diffusion" or "surface" techniques, based on how the antibody probes arrive at their targets. Despite some overlap, this distinction is meant to indicate that in the former, antibodies are allowed to diffuse into and out of the fixed tissue, whereas in the latter the tissue is first sectioned on an ultramicrotome, and the antibodies are subsequently bound to antigenic sites exposed mainly on the surface of the section.

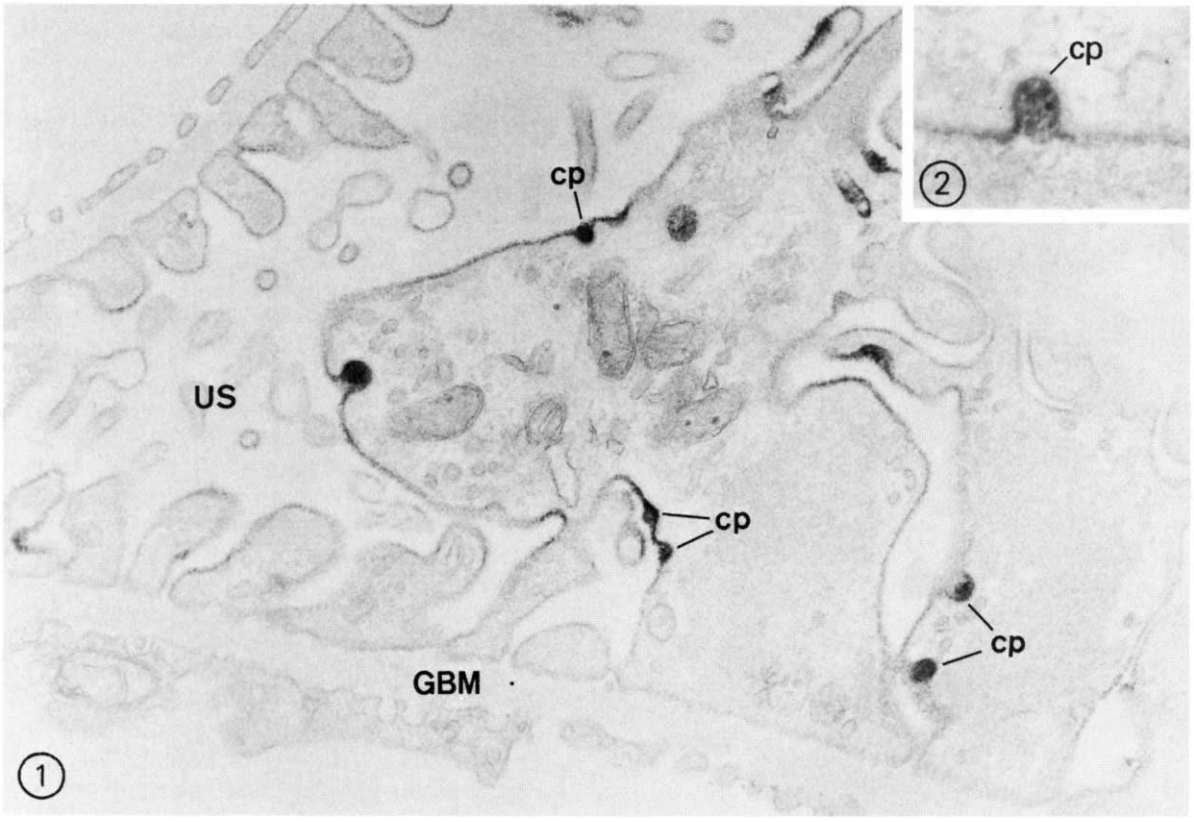
Most of the methods referred to here are "indirect" immunocytochemical procedures, in which a given antigenic molecule is first complexed with an unlabeled primary antibody, followed by incubation in a labeled secondary antibody specific for the species in which the primary antibody was raised.

Diffusion methods

A typical procedure involves the following steps: 1) fixation of the tissue by chemical crosslinking with aldehydes; 2) sectioning of the samples either without freezing on a Vibratome, or on a cryostat after incubation in a cryoprotective agent and freezing in liquid N_2; 3) incubation with the primary antibody (IgG, affinity purified IgG, or monoclonal IgG); 4) incubation with a secondary labeled probe (usually peroxidase conjugated to IgG fragments or to protein A, or colloidal gold coupled to IgG or protein A); 5) stabilization of the immune complexes and tissue components by crosslinking with glutaraldehyde; 6) visualization of peroxidase activity by the diaminobenzidine (DAB) reaction (in the case of peroxidase conjugates); 7) osmication and embedding in epoxy resins from which ultrathin sections are prepared for examination in the electron microscope. Details of these procedures are in [10–17].

Surface methods

The most common protocols are postembedding methods that call for: 1) aldehyde fixation of the tissue samples; 2)



Figs. 1 and 2. *Localization of gp330, the pathogenic antigen of Hey-mann nephritis, by indirect immunoperoxidase in glomeruli of normal Lewis rats.* Cryostat sections of PLP-fixed kidneys were incubated with polyclonal (rabbit) anti-gp330 IgG, followed by sheep anti-rabbit Fab-peroxidase conjugate which was detected by the diaminobenzidine reaction. Gp330 is found exclusively in visceral glomerular epithelial cells where it is concentrated in coated pits (cp) located both on the surface of the cells facing the urinary spaces (US), and that facing the glomerular basement membrane (GBM) (Fig. 2). (Fig. 1, × 21,000; Fig. 2, × 34,000.)

dehydration and embedding in epoxy or acrylic resins (such as Lowicryl [18, 19], or LR-White [20]); 3) preparation of ultrathin sections which are mounted on grids; 4) incubation in primary antibodies and secondary labeled probes as for diffusion methods followed by examination in the electron microscope.

Alternatively, ultrathin cryosections can be cut directly from tissue frozen at liquid N_2 temperature on an ultramicrotome equipped with a cryoattachment, mounted on grids, followed by immunolabelling and staining. Details of these procedures are in [21–26].

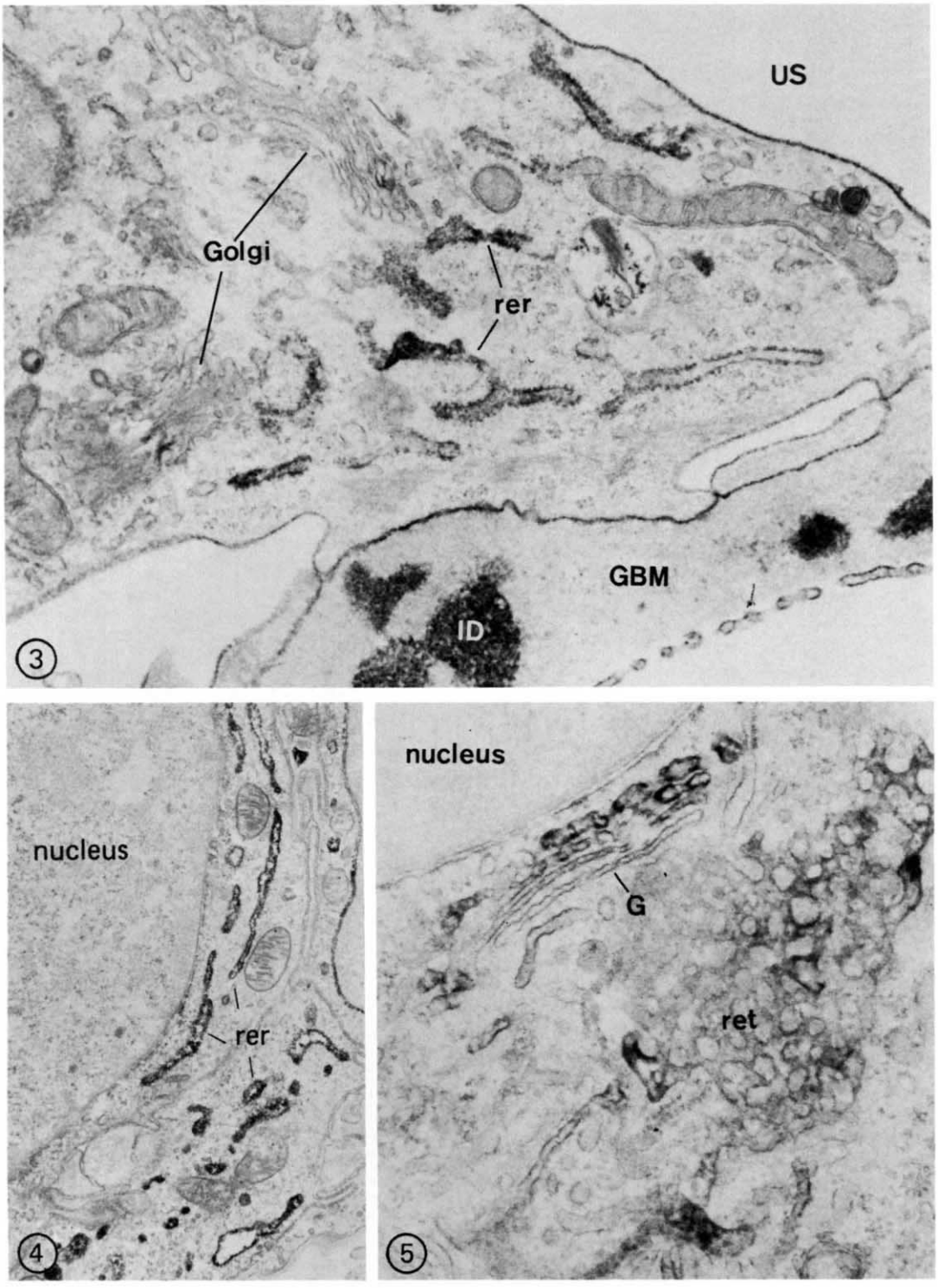
Some pros and cons of immunoelectron microscopical methods

Each of the currently available procedures has several benefits as well as certain disadvantages which must be considered before a method is selected.

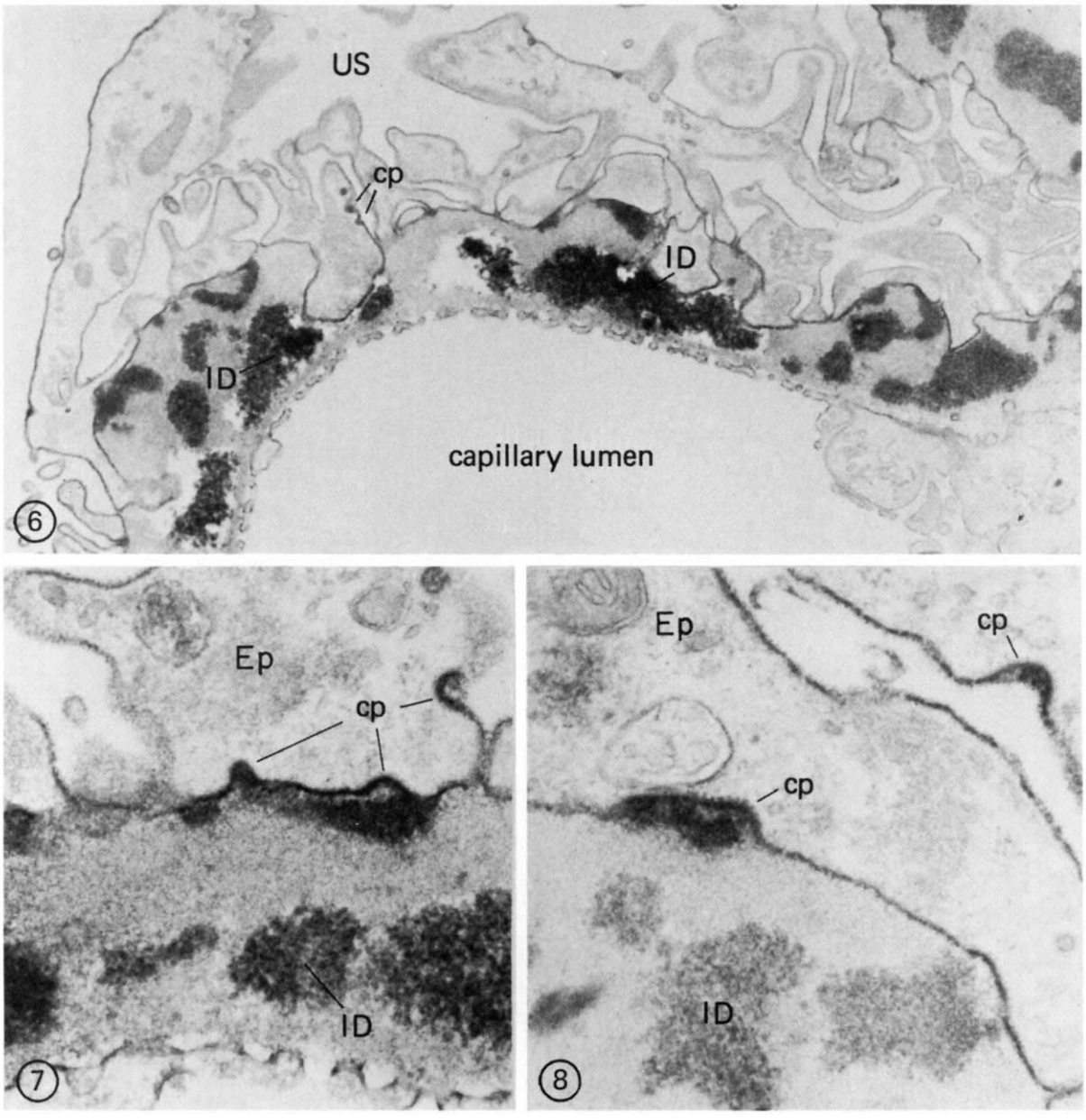
Immunoperoxidase was among the first and most commonly applied immunoelectron microscopical techniques. It is unsurpassed in sensitivity. Recently, greatly improved procedures of fixation and permeabilization [10, 13, 27] have been introduced that permit very good preservation of morphological structure (Figs. 1–9). In addition, immunoperoxidase is relatively simple to perform and does not require any special equipment. Because a histochemical enzyme reaction is used for the visualization of the antigen, the amount of reaction product can be varied by limiting or extending the time of the reaction. This is the reason for the high sensitivity of this method, but it may also be the cause of a major hazard, because the DAB reaction product can diffuse away from its site of generation and bind to adjacent cell structures [28].

Surface methods in combination with immunogold procedures avoid the diffusion problems encountered with immunoperoxidase, but they also have limitations. The most serious is their relatively lower sensitivity which is due to the fact that only a limited number of antigenic molecules are exposed on the surface of the section, when compared with the greater amounts of antigen which are available for antibody binding in diffusion methods.

The least satisfactory material for surface localization in tissue is embedding in hydrophobic resins, such as Epon 812, because the embedding process partially or completely denatures many antigens and frequently obscures antigenic sites on the surface of the ultrathin section. Some antigens can be exposed by partial solubilization ("etching") of the resin [29], but the antigenicity of many molecules does not survive the necessary extreme conditions. As an alternative for embedding of tissue, more hydrophilic acrylic resins—such as Lowicryl



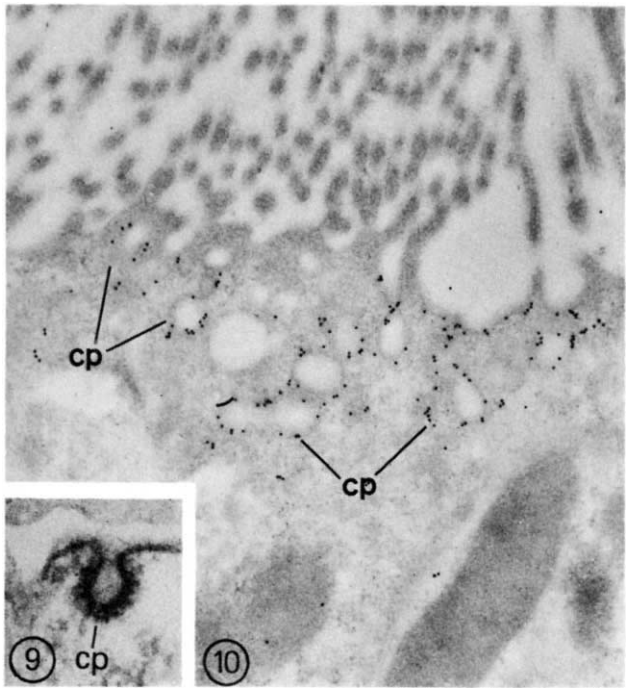
Figs. 3–5. *Examples of the localization of gp330 in intracellular compartments by indirect immunoperoxidase.* All samples were prepared as described in the preceding figures. **Fig. 3.** Glomerulus of a rat with advanced active Heymann nephritis reacted with affinity purified rabbit anti-gp330 IgG and sheep anti-rabbit-Fab peroxidase conjugate. Gp330 is found in the cisternae of the rough endoplasmic reticulum (rer) indicating that it is synthesized by this epithelium. The antigen is also detected in immune deposits (ID) in the GBM. **Fig. 4.** Rat visceral yolk sac epithelium, reacted with monoclonal anti-gp330 IgG and sheep anti-mouse Fab-peroxidase conjugate. The endoplasmic reticulum (rer) of this epithelium also contains gp330. **Fig. 5.** Localization of gp330 in the epithelium of the proximal tubule of a rat which was made proteinuric by injections of aminonucleoside of puromycin for eight days. The DAB-reaction product is concentrated in two of the stacked cisternae of the Golgi complex (G), and it is also found in a reticular cisterna (ret) adjacent to the Golgi stacks. (Fig. 3, × 32,000; Fig. 4, × 10,000, Fig. 5, × 32,000.)



Figs. 6–8. *Localization of gp330 in the immune deposits (ID) of rats with advanced active HN, detected by indirect immunoperoxidase with affinity purified rabbit anti-gp330 IgG and sheet anti-rabbit Fab-peroxidase conjugate.* Cryostat sections of PLP-fixed HN-nephritis rat kidneys were treated as described in Figure 1. In all micrographs, gp330 is observed in immune deposits (ID) in the GBM. Several of the immune deposits (**Figs. 7 and 8**) are associated with coated pits (cp) present on the basal cell membrane of the visceral epithelial cells (Ep). (Fig. 6, × 21,000; Figs. 7 and 8, × 36,000.)

K4M (a low–temperature embedding medium) [18] and LR White [20]—were developed, and have proved to be very useful for the localization of several antigens [19, 20, 30, 31] (see Figs. 10 and 17), and lectin–binding sites [32–34] in the kidney as well as other tissues. Advantages of the acrylic resins are the simplicity of the immunostaining procedure on sections, and the possibility of collecting and storing large numbers of tissue blocks (such as from human biopsy material) which can be subsequently used for retrospective immunocytochemical investigations. However, in our experience some antigens, particularly membrane proteins that are present in relatively small amounts, are not demonstrable in these preparations but are detectable by other techniques.

Ultrathin frozen sectioning offers an alternative procedure that avoids complications introduced by the usual plastic embedding procedures. Recently, the successful immunocytochemical localization of various antigens (such as viral and receptor glycoproteins [35, 36]) on ultrathin frozen sections has demonstrated the great potential of this procedure, in which both antigenicity and morphology are reasonably well pre-



Figs. 9 and 10. *Localization of clathrin in the brush border of the proximal tubule epithelium with affinity–purified anti-clathrin IgG.* In Figure 9 an indirect immunoperoxidase (diffusion) method as described in Figure 1 was used. In Figure 10, an ultrathin section of PLP-fixed rat kidney which had been embedded in the acrylic resin LR-White was incubated in anti-clathrin IgG, followed by a goat anti-rabbit IgG gold conjugate (10 nm). In both cases, clathrin was found on the cytoplasmic aspect of the coated invaginations of the cell membranes located at the base of the microvilli in the brush border. (Fig. 9, × 15,000; Fig. 10, × 42,000.)

served (see Figs. 23–25). A disadvantage of this technique is that, at the present time, special equipment (cryoattachments) and considerable skill and experience are required to obtain reliable results consistently.

A valuable byproduct that cryosectioning has contributed to the immunohistochemical repertoire is the ability to readily cut 0.5 to 1 μm "semithin" sections, on which immunofluorescence and immunoperoxidase techniques can be performed with high resolution [11, 15, 22, 37]. In these sections the association of antigens and specific organelles can often be deduced. For example, the distinctive distribution of endosomal and lysosomal membrane proteins in the proximal tubule cell was distinguished in such sections using double labeling procedures evaluated at the light microscope level [15].

From the foregoing, it should be evident that the shortcomings of surface and diffusion techniques complement each other to a certain extent. Therefore, for greatest confidence in the results it is desirable to confirm independently the results obtained by one method with an alternative, complementary procedure.

Problems common to all procedures

Fixation

Fixation of tissues with 1 to 3% glutaraldehyde in various buffers by vascular perfusion results in good preservation of morphology, but it frequently destroys partially [38] or completely [13] the antigenicity of proteins. On the other hand, formaldehyde solutions (freshly prepared from paraformaldehyde) preserve antigenicity much better than glutaraldehyde but stabilize tissues less satisfactorily. An ideal compromise for many antigens (such as gp330) [38], is a mixture of low concentrations of glutaraldehyde (0.05 to 0.1%) in combination with 4% formaldehyde applied by perfusion to kidneys for a short time (5 to 10 min). For antigens which are particularly glutaraldehyde sensitive, a formaldehyde fixative containing lysine and periodate (PLP) [39], has been used successfully [11, 13–16]. After fixation with PLP by vascular perfusion there is good preservation of structure as well as antigenicity, and good permeability of cells to peroxidase conjugates.

After fixation, some authors use compounds containing amino groups, such as glycine and ammonium ions, to quench free aldehyde groups and to prevent nonspecific sticking of antibodies. This step appears to be important for glutaraldehyde–fixed tissues, but in our hands it has not been found to be essential when using formaldehyde–containing fixatives, such as PLP.

Characterization and purification of primary antibodies

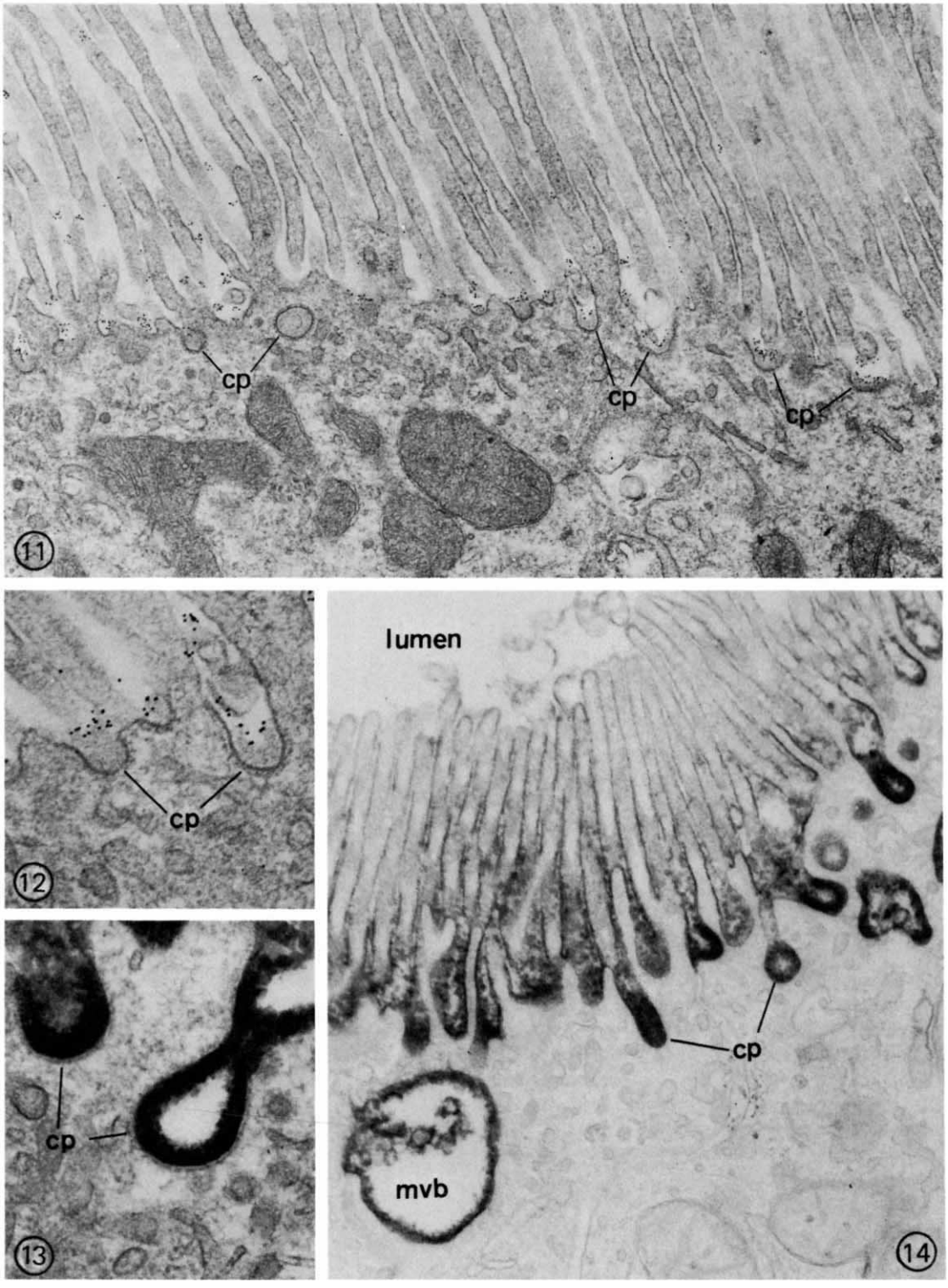
Before undertaking immunoelectron microscopy, it is essential to determine the specificity of the primary antibody to be utilized and to demonstrate that it recognizes a single protein. For this purpose, several sensitive immunochemical methods are available, such as immunoprecipitation of radioactively labeled antigen [40, 41], and immunoblotting (that is, separation of proteins by SDS-PAGE followed by their transfer onto nitrocellulose and immuneoverlay with appropriate antibody reagents [42, 43]).

Since the use of whole serum sometimes results in high "background staining", even when the titer of the specific antibody is high, it is advantageous to prepare purified IgG or affinity purified IgG. The affinity purification can be accomplished by binding IgG to a solid support, such as cyanogen bromide-activated Sepharose [38], or to nitrocellulose paper [44] to which the antigen has been immobilized, followed by elution of the bound antibody. Binding to nitrocellulose paper is the simplest procedure and produces enough affinity purified IgG to satisfy most needs [15, 17, 20].

When monoclonal antibodies are used, the monospecificity of the probe is usually not a matter of concern, although some unexpected crossreactivities, for example, crossreactivity of IgG specific for a membrane protein with components of the cytoskeleton, have been reported [45]. However, because only a single epitope is recognized, usually a single IgG molecule will bind per molecule of antigen and, accordingly, the immunohistochemical signal obtained may be weak when compared to that seen with polyclonal antibodies. One way to increase the antibody/antigen ratio is to mix together several monoclonal IgGs which are directed against different epitopes of the same molecule [38].

Route of application of primary antibodies

Usually the primary antibodies are incubated with tissue sections, that is ultrathin plastic–embedded or frozen sections in the case of surface techniques, or cryostat or Vibratome



Figs. 11–14. Selective localization of gp330 in coated pits (cp) in the proximal tubule brush border by both indirect immunogold (Figs. 11 and 12) and immunoperoxidase (Figs. 13 and 14) procedures. Cryostat sections of PLP-fixed rat kidney cortex were incubated in monoclonal anti-gp330 IgG, followed by a goat anti-mouse IgG gold (10 nm) conjugate (Figs. 11 and 12) or a sheep anti-mouse Fab HRP conjugate (Fig. 13). Figure 14, from a similarly prepared human kidney, was incubated with affinity–purified rabbit anti-rat gp330 IgG, and sheep anti-rabbit Fab-HRP conjugate. In all examples, gp330 is found concentrated in clathrin coated pits (cp) at the base of the microvilli and is occasionally seen in multivescular bodies (mvb). (Fig. 11, × 18,000; Figs. 12 and 13, × 38,000; Fig. 14, × 19,000.)

sections in the case of diffusion methods, which contain the antigen of interest.

In some cases, as an alternative, the antibody can also be administered intravenously in vivo. This technique has been applied to several antibodies raised against GBM components such as laminin [46] and heparan sulfate proteoglycans [9, 47]. The usefulness of this approach in the localization of renal antigens is limited mainly to glomerular antigens (GBM and endothelial) that are directly exposed to the circulation.

Secondary probes labeled with electron dense markers.

To detect the first antibody, frequently affinity purified IgG or Fab fragments prepared therefrom are applied which are specific for the IgG of the species in which the first antibody had been raised. These probes usually do not crossreact with IgG from other species. An exception is the case of mouse and rat IgG which may crossreact and create a problem when antigens are detected with monoclonal mouse IgG in rat tissues. Adsorption of the secondary IgG on immobilized purified rat IgG results in efficient elimination of the undesired crossreactive IgG component.

Protein A can be used as an alternative to IgG when the primary antibody belongs to a protein A-binding class—such as rabbit IgG and some monoclonal mouse IgGs.

These secondary probes are conjugated to an electron–dense tracer such as horseradish peroxidase, colloidal gold or ferritin, which render them visible in the electron microscope.

Conjugates with horseradish peroxidase (HRP)

HRP can be readily detected at the electron microscope level by virtue of a simple peroxidase reaction (involving the generation of oxidized, polymerized DAB) [48]. This property is exploited for the localization of antigens by using specific antibodies conjugated to the enzyme [49]. A major advantage of this electron dense marker is that it is relatively small (Mr = 45,000) in comparison to gold or ferritin, and accordingly, the conjugates can be made very small, for example by conjugating HRP to Fab fragments (Mr = 17,000), thus facilitating their diffusion into tissue.

When the conjugated antibody has bound to its target, DAB reaction product can be generated by the DAB reaction. This results in considerable amplification of the signal and ensures the high sensitivity of the method, but in some cases (depending on the location of the antigen), it can also create a problem in the precise localization of an antigen because diffusion and relocation of the DAB onto adjacent structures (such as membranes) can occur during the incubation procedure [28] (Figs. 20–22).

Gold conjugates

When IgG or protein A are adsorbed to the surface of colloidal gold particles [50] probes are generated which have been crucial for the development and success of surface techniques [30, 31, 35]. Among the several advantages of these probes is: 1) their availability in different, well defined sizes for simultaneous visualization of two different antigens in the same section; and 2) the option to quantitate the density of an antigen by counting the number of gold particles localized to a given structure [15, 17, 51]. Gold conjugates can also be used for diffusion methods when the antigen is exposed on the surface of cells (see Figs. 11, 12 16 and 18) [8, 17, 51] or cell fractions [15]. However, because of their large size (5 to 15nm) gold conjugates do not readily penetrate into the interior of cells, and for that reason they have not proved to be very useful for the localization of intracellular antigens by diffusion methods.

Several other conjugates of secondary probes and electron dense marker molecules, such as ferritin [52] have also been used successfully. Ferritin has the advantage that, being a protein, it can be covalently bound to the antibody, but it is less electron dense than colloidal gold and is available only in one size.

Biotin–avidin systems

As an alternative to antibodies as secondary probes, avidin– and stepavidin–biotin systems have been adopted for immunoelectron microscopy [53]. The procedure takes advantage of the high avidin–biotin binding affinity. Often the first antibodies are labeled with biotin and the secondary probe (ferritin, gold, HRP) is coupled to avidin, or, alternatively, a three step procedure (unlabeled first antibody plus biotinylated second antibody and avidin–ferritin, gold or HRP) is used. An advantage is the high sensitivity of such detection systems [1, 54] which theoretically, at least, equals or surpasses that of Fab-HRP conjugates as secondary probes. Due to their high sensitivity avidin–biotin–HRP systems are used increasingly for immunocytochemistry at the light microscopic level. So far, they have been used less frequently for immunoelectron microscopy.

Accessibility of the immunocytochemical probes

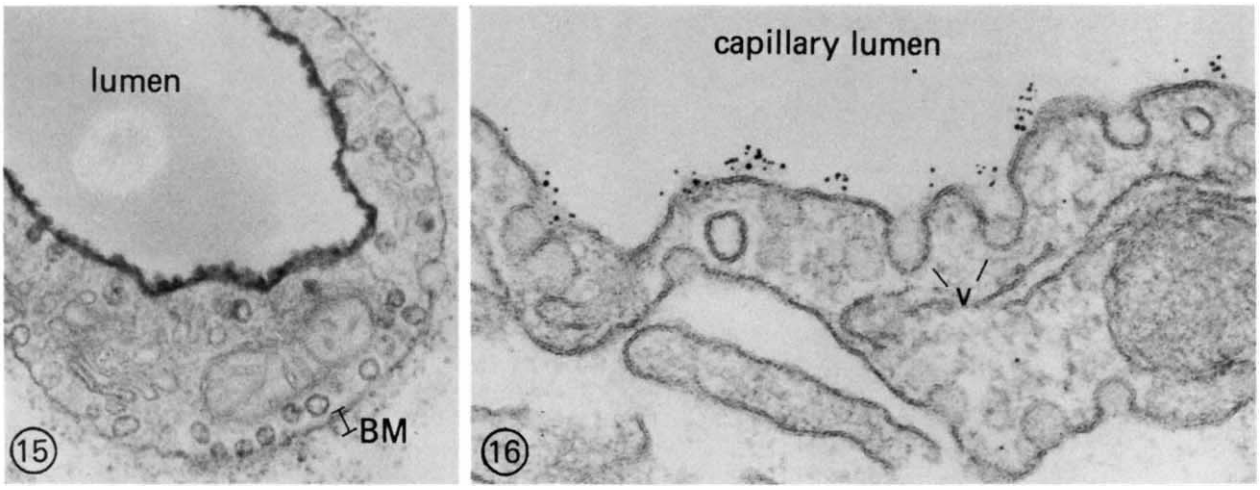
With surface methods one depends on the labeling of antigenic determinants exposed on the surface of the sections. With diffusion methods, however, one depends on penetration of antibodies into various cell compartments, and their penetration is limited by the cell membrane as well as the membranes of intracellular compartments, such as those of the endoplasmic reticulum, Golgi complex, and lysosomes. It is therefore necessary to permeabilize these membranes for the immunoprobes to gain access to their antigens. Adequate permeabilization of tissues is achieved by freezing the specimens in the presence of cryoprotectants such as 10% DMSO and sectioning on a cryostat at −20°C. Cultured cells are frequently permeabilized by treating them with low concentrations of detergent (such as saponin, 0.05 to 0.1%) for electron microscopy [55] or Triton X-100 for light microscopy.

Gold conjugates are usually too big to gain access to intracellular antigens unless the membranes are solubilized, for example, by extraction with Triton X-100, but this treatment usually results in extensive disruption of cell architecture. So far this approach has been used satisfactorily only for the localization of components of the cytoskeleton [56].

Contributions of immunoelectron microscopy to the understanding of glomerular problems

Studies on Heymann nephritis

In a series of studies carried out in the last four to five years, we have identified and characterized the brush border glycoprotein gp330 [7, 38, 57] and have provided evidence that it is the major—probably the sole—antigen of Heymann nephritis



Figs. 15 and 16. Localization of podocalyxin on endothelial cells of rat heart. Comparison of the resolution obtained by the indirect immunoperoxidase (Fig. 15) and indirect immunogold (Fig. 16) diffusion procedures. Cryostat sections of PLP-fixed rat heart were first incubated in affinity purified rabbit anti-rat podocalyxin IgG, followed by either a sheep anti-rabbit Fab-peroxidase conjugate (Fig. 15), or a goat anti-rabbit IgG coupled to colloidal gold (10 nm) (Fig. 16). The DAB-reaction product obtained by immunoperoxidase is evenly distributed on the luminal membrane of the endothelial cell; however, the immunogold labeling reveals a patchy distribution of podocalyxin sparing most of the invaginating endothelial vesicles (v). (Figs. 15 and 16, × 25,000.)

(HN). This finding has recently been confirmed by several other groups [1, 54, 58–62]. The identification of gp330 as the major pathogenic antigen in this disease model was done initially by demonstrating that: 1) IgG which had been eluted from immune deposits of kidneys with active HN specifically immunoprecipitates only this molecule [57]; 2) purified gp330 induces active HN [57], and 3) an antibody specific for gp330 induces passive HN, that is, it binds to glomeruli when injected intravenously [38]. Several questions arose from these findings, and immunohistochemistry proved to be crucial to finding the answers.

A central question concerned the basis for the shared nature of the pathogenic antigen in proximal tubule brush borders and in glomeruli, where it would be expected to be found from the "in situ" hypothesis for the formation of immune deposits [63, 64]. The localization of gp330 by immunofluorescence confirmed that it is present in both sites, but the reason for this dual localization was not apparent. When gp330 was localized in glomeruli at the electron microscopical level with monoclonal anti-gp330 IgG, it was found to be an epithelial antigen, rather than a basement membrane component as had been often assumed [63, 65], because it was localized exclusively in coated pits at the cell surface and in biosynthetic compartments (rough ER and Golgi cisternae) of podocytes [38] (Figs. 1–3). When gp330 was similarly localized in the proximal tubule brush border, it was found to be concentrated in clathrin-coated pits [17] at the base of microvilli (Figs. 11–14) and was not found on the microvillar membranes themselves [11]. Thus, the fact that gp330 is concentrated in coated pits in both proximal tubule and glomerular epithelia provided a logical explanation for the common presence of gp330 in these two cell types.

Since coated pits are well known to be involved in receptor mediated endocytosis, the localization of gp330 in coated pits raised the question of whether it is an endogenous antigen or an exogenous ligand undergoing adsorption. Here again, immunoelectron microscopy provided valuable information because gp330 was detected in biosynthetic compartments (ER and Golgi) of visceral glomerular epithelial cells [38] (Fig. 3). Along with complementary biosynthetic data on isolated glomeruli after radiolabeling [38], this clearly indicated that gp330 is an endogenous glomerular component which is produced by epithelial cells, rather than an exogenous molecule absorbed from the circulation. In fact, gp330 appears to be absent from the circulating blood, whereas another protein, maltase, which is structurally related to gp330 but does not induce HN, is present in the plasma in high concentrations (unpublished data).

A survey of various organs by immunoelectron microscopy revealed that gp330 is not a universal component of all coated pits, but it is found in epithelia of several other organs, that is, epididymis [12], yolk sac (Fig. 4) [12], and pneumocytes type II [1]. In all these epithelia, gp330 was found to be restricted to coated pits, usually exposed on the luminal or absorptive surface of the cells, and to biosynthetic compartments. In addition, gp330 was detected in renal proximal tubules of several different species, such as the mouse [66] and, interestingly, in humans (Fig. 14) (unpublished data). It is also present in cultured visceral glomerular epithelial cells obtained from rats [67]. These findings indicate that gp330 or a family of gp330-like glycoproteins is widespread among organs and species. The fact that it is found predominantly in absorptive epithelia suggests that these molecules represent either receptors for which the ligands are unknown to date, or constitutive components of a subclass of coated pits.

The use of monoclonal antibodies for immunocytochemical localizations in the proximal tubule cell also enabled us to distinguish between two proteins, gp330 and the enzyme maltase, which share epitopes in common (by peptide mapping) [11], and thus could not be discriminated by polyclonal antibodies. By applying monoclonal antibodies which specifically recognize either gp330 or maltase, we found by immunocy-

tochemistry that these two glycoproteins are localized in different microdomains of the proximal tubule brush border: gp330 is concentrated on the membranes of the intermicrovillar clathrin-coated pits (Figs. 11–13), whereas maltase is found on the membranes of the microvilli themselves [11, 15, 17]. This differential distribution was demonstrated by both immunoperoxidase and immunogold techniques (Figs. 11–13) and provided the first evidence that the brush border membranes of proximal tubules contain microdomains of distinctive glycoprotein composition.

Immunoelectron microscopy has also provided valuable insights into how immune deposits are formed in HN. In animals examined three days after injection of anti-gp330 IgG, the injected IgG was detected in coated pits [38], suggesting that the coated pits could be the site where the antigen and the antibody meet. Recently, immunoelectron microscopy has been used to follow the very early steps in the formation of immune deposits in HN. In these studies immunoperoxidase was indispensible because of its high sensitivity. Small amounts of anti-gp330 IgG could be found in coated pits at the base of the foot processes of the visceral glomerular epithelium as early as 10 minutes after intravenous injection of the antibody. With time, these deposits increased in size and in number and usually were not found in association with coated pits. However, by serial sectioning we have found that virtually every one of these large immune deposits is associated on part of its surface with a coated pit (Kerjaschki, Miettinen and Farquhar, unpublished data). Also, in advanced active HN immune deposits are found in association with coated pits (Figs. 6–8). These findings suggest that immune deposits arise by repeated binding of anti-gp330 IgG to its antigen in coated pits, after which the immune complexes are shed into the lamina rara externa of the GBM and accumulate there.

Studies on podocalyxin

Podocalyxin (apparent Mr = 140,000) is the major sialoglycoprotein of the glomerulus [7]. We previously provided evidence that this molecule is the major component of the glomerular epithelial polyanion which has been defined by histochemistry [68–70], and of which tryptic peptides have been prepared [71]. It is the only glomerular component that stains with the polycationic dyes "stains all" and alcian blue in SDS-gels, and binds wheat germ agglutinin on nitrocellulose transfers (lectin–blots) [7]. Staining for "glomerular polyanion" is known to be reduced or lost in several glomerular diseases [72, 73]. Direct chemical analysis of the sugar composition of podocalyxin indicated that in puromycin nephrosis of the rat (a disease model for human minimal change nephrosis), the sialic acid content of the podocalyxin molecule is reduced from 15 to 4 to 5 sialic acid residues, whereas other saccharides remain unchanged [51].

Results of localization of podocalyxin by immunofluorescence confirmed that it is found on glomerular epithelial cells (Figs. 17, 18). It was also detected on endothelial cells, but was not found on any other type of epithelial cell in the kidney or other tissues [7]. Immunoelectron microscopy carried out by immunoperoxidase or immunogold (Figs. 17–18) procedures on cryostat sections indicated that this molecule is present on the surface of glomerular epithelial cells [7, 20], that is, in precisely the same location in which the "glomerular polyanion" has been found previously by staining with cationic electron dense markers, such as colloidal iron [69, 70, 74]. A question that arose is whether podocalyxin is present on the base as well as the sides of the foot processes. This is the type of question that can be answered only by immunoelectron microscopy. However, it could not be answered reliably by diffusion methods, because access to the antigens in the region of the cleft between GBM and plasmalemma of the epithelial cell is limited due to the restricted permeability of the GBM. However, such antigens are often accessible by surface labeling procedures. When immunogold reagents were used in surface techniques [75], it was apparent that podocalyxin was either absent from this region, or present only in small amounts below the sensitivity of the method (Fig. 17).

Recently, podocalyxin was also found to be a widespread if not a general component of endothelial cell membranes (Figs. 15, 16) [20]. Here again, immunoelectron microscopy was invaluable for determining the domains and microdomains of the endothelial plasmalemma on which podocalyxin is concentrated. It was found by immunogold procedures to be restricted to the luminal domain of the endothelium of lung capillaries—where its presence was also confirmed by immuneoverlays—and of various other blood vessels where it was localized in a patchy pattern [20].

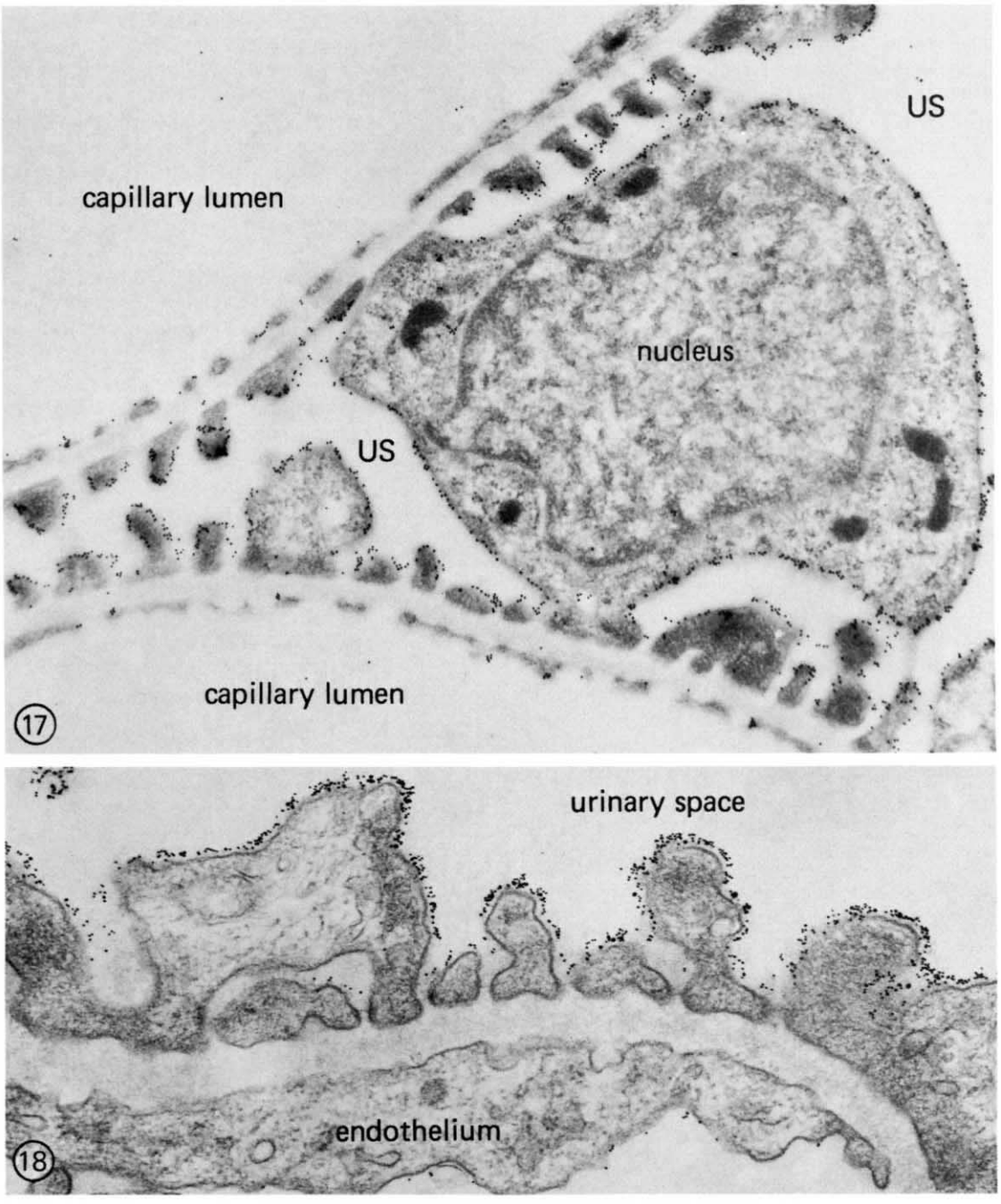
Localization of components of the glomerular basement membrane

Immunoelectron microscopy has been very valuable for establishing the presence of various extracellular matrix antigens such as laminin, collagens and proteoglycans, in the GBM and in other basement membranes [9, 10, 76–85] (Table 1). At the EM level, successful localizations have been achieved in intact tissues and in isolated GBM preparations using both diffusion and surface techniques. Thus, the results are satisfying in that they have provided corroborative and convergent information indicating that laminin, type IV collagen and heparan sulfate proteoglycans are integral components of the GBM. The question of whether or not fibronectin is a true component of the GBM is still controversial [10, 76, 82, 84].

Attempts to utilize immunoelectron microscopy to establish the localization of these constitutive molecules more precisely within the various layers (lamina rara interna, lamina densa, lamina rara externa) of the GBM have in general not been very useful or satisfying because, as shown in Table 1, they have yielded conflicting results. This is undoubtedly due in part to the different antibodies used and the different approaches followed, each with its own inherent limitations. However, it is also due to the fact that the immunocytochemical localization of antigens within the GBM has inherent problems in that not all GBM layers are equally accessible to antibodies, and that some antigenic sites may be masked [86–88] due to the highly cross-linked nature of the GBM.

Results obtained by diffusion methods.

Diffusion methods have been most widely used for immunolocalization of GBM antigens, and they have been quite helpful in establishing the presence of specific extracellular matrix components in the GBM and in other basement membranes. However, as already indicated, problems arise when



Figs. 17 and 18. *Localization of podocalyxin in the rat glomerulus by surface (Fig. 17) and diffusion (Fig. 18) procedures.* Kidneys for both examples were fixed by perfusion with PLP. In Fig. 17, the tissue was embedded in LR-White acrylic resin, and ultrathin sections were prepared and incubated with affinity purified rabbit–anti-podocalyxin IgG and 10 nm gold goat anti-rabbit IgG. Gold particles heavily outline the entire plasma membrane of the visceral glomerular epithelial cell except for those regions along the base of the foot processes facing the GBM. A lower concentration of gold particles is also seen along the endothelial cell membrane. Of note is the fact that in this type of preparation which does not depend on diffusional access to antigens (because they are exposed on the surface of ultrathin sections) there is no labeling of the "soles" of the foot processes, indicating that podocalyxin is absent or present in very low concentrations on this microdomain of the epithelial plasmalemma. In Fig. 18, cryostat sections were treated as described in Fig. 11. The distribution of gold particles is the same as in Fig. 17. However, from this type of preparation alone, it cannot be decided whether or not podocalyxin is present at the base of the foot processes, because the gold conjugate is too large to freely diffuse into this area. US = urinary space. (Fig. 17, × 16,500; Fig. 18, × 20,600.)

trying to asign the localization of any given antigen to a specific layer or structural component of the GBM. The first problem to be dealt with is the fact that not all regions of the GBM are equally accessible by diffusion. Antibodies, including ferritin [84] and gold conjugates, can gain access easily to the lamina rara interna because the endothelium of glomerular capillaries is "open" in that it is fenestrated and lacks diaphragms. Accordingly, there is a barrier between it and the capillary lumen.

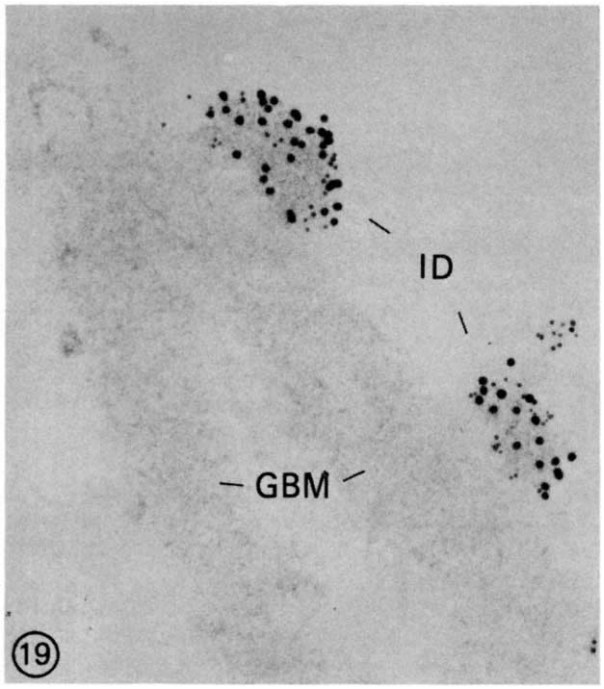


Fig. 19. *Double-localization of gp330 and rabbit IgG in an isolated GBM preparation of a rat sacrificed three days after intravenous injection of rabbit anti-gp330 IgG.* The glomeruli were isolated by sieving, and cells were lysed by detergent treatment. The isolated GBMs were fixed in PLP, attached to a glass surface with poly-1-lysine and incubated in monoclonal anti-gp330 IgG followed by 5 nm gold goat anti-mouse IgG (for the visualization of gp330), and 10 nm gold anti-rabbit IgG (for the visualization of rabbit anti-gp330 IgG). Note that the immune deposits (ID) remain associated with the GBM, and both small and large gold particles can be detected in these deposits indicating the presence of both the antigen and the anti-gp330 antibody.

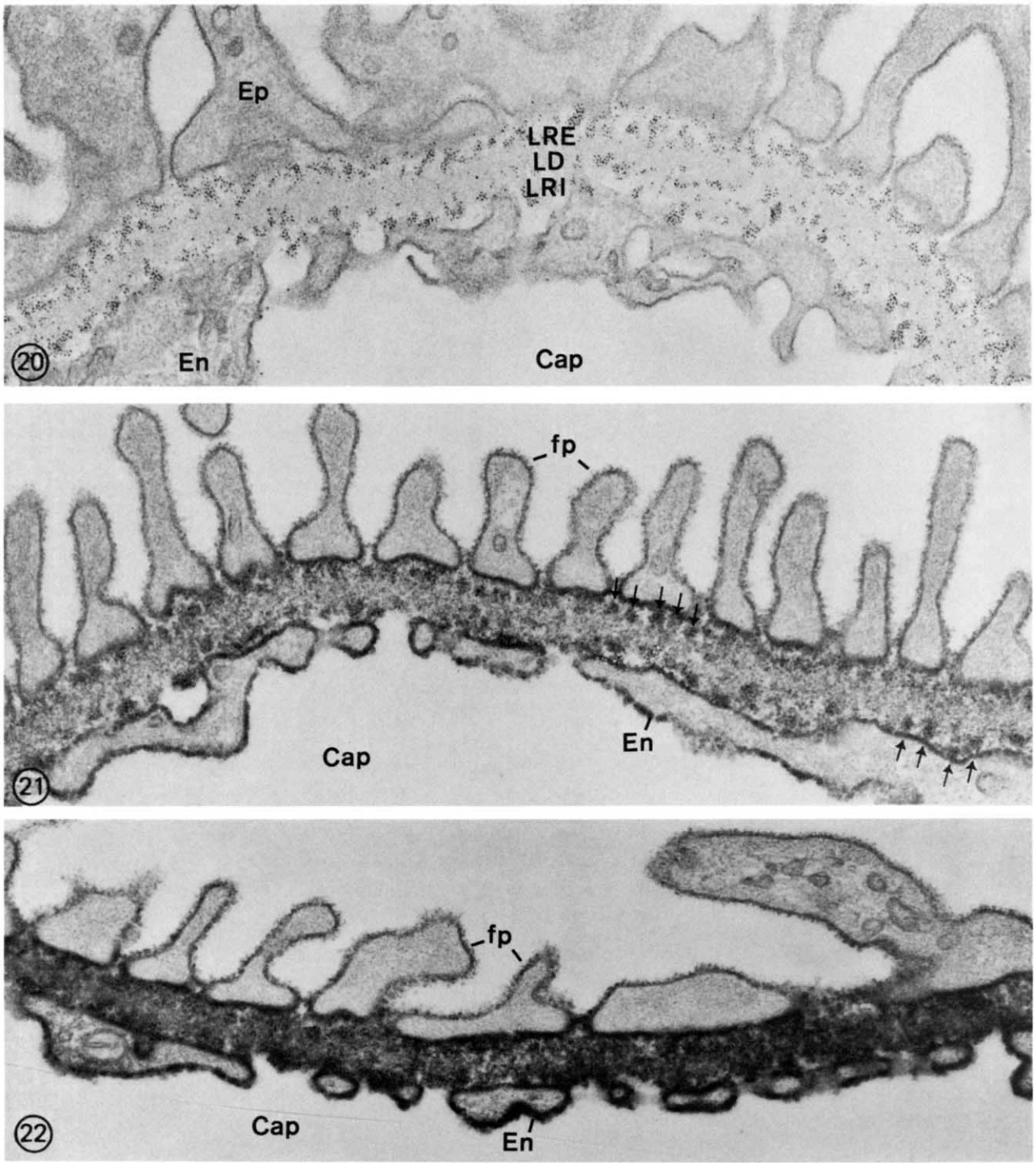
However, an increasingly steep gradient to diffusion (LRE > LD > LRI) exists across the GBM due to its special properties as the main filtration barrier for anionic [89, 90] and neutral [91] macromolecules. In our experience [84], slit diaphragms are also impermeable to antibody conjugates which prevents access by diffusion from the urinary spaces. Therefore, the area between the lamina densa of the GBM and the slit diaphragms of the podocytes is the most difficult region of the GBM for macromolecular probes to gain access by diffusion. Several approaches have been devised to get around this problem: 1) to reduce the size of the reagents to make them as small as possible, that is, by using small probes, such as HRP coupled to Fab fragments of IgG [9, 10, 84]; 2) pretreatment of tissue blocks or cryostat sections with a reducing agent such as sodium borohydride [76, 77, 80]; or 3) use of GBM fractions [10, 84] (Fig. 19). Digestion of tissue blocks or cryostat sections with collagenase has also been tried (unpublished observations) and seems to have some potential [92].

The next problem confronted, as mentioned earlier, is the fact that the use of HRP conjugates introduces the possibility of generating DAB diffusion artifacts. The existence of this problem was graphically illustrated in a model system in which a foreign antigen (cationized ferritin) was implanted in the lamina rara interna and lamina rara externa of the GBM [93] (Fig. 20), where it bound to the heparan sulfate proteoglycans [94], and an anti-ferritin antibody was used to localize the bound ferritin [28]. Under limited reaction conditions (use of low concentrations of primary antibody and brief exposure to DAB), reaction product was confined mainly to the laminae rarae where the cationized ferritin was located (Fig. 21). However, even under minimal reaction conditions, some reaction product was seen on the adjoining epithelial and endothelial cell membranes, and under prolonged incubation conditions it was deposited throughout all layers of the GBM (Fig. 22). These findings demonstrate that DAB can diffuse over long distances (50 to 100 nm) and, accordingly, the reliable resolution of immunoperoxidase method for detection of GBM antigens is limited to the GBM as a whole. Therefore, interpretation of the localization of antigens in the various GBM layers should be approached with caution. For greatest confidence in the results, they should be checked against those obtained with alternative approaches, such as surface labeling or a direct immunoperoxidase technique after in vivo antibody injection.

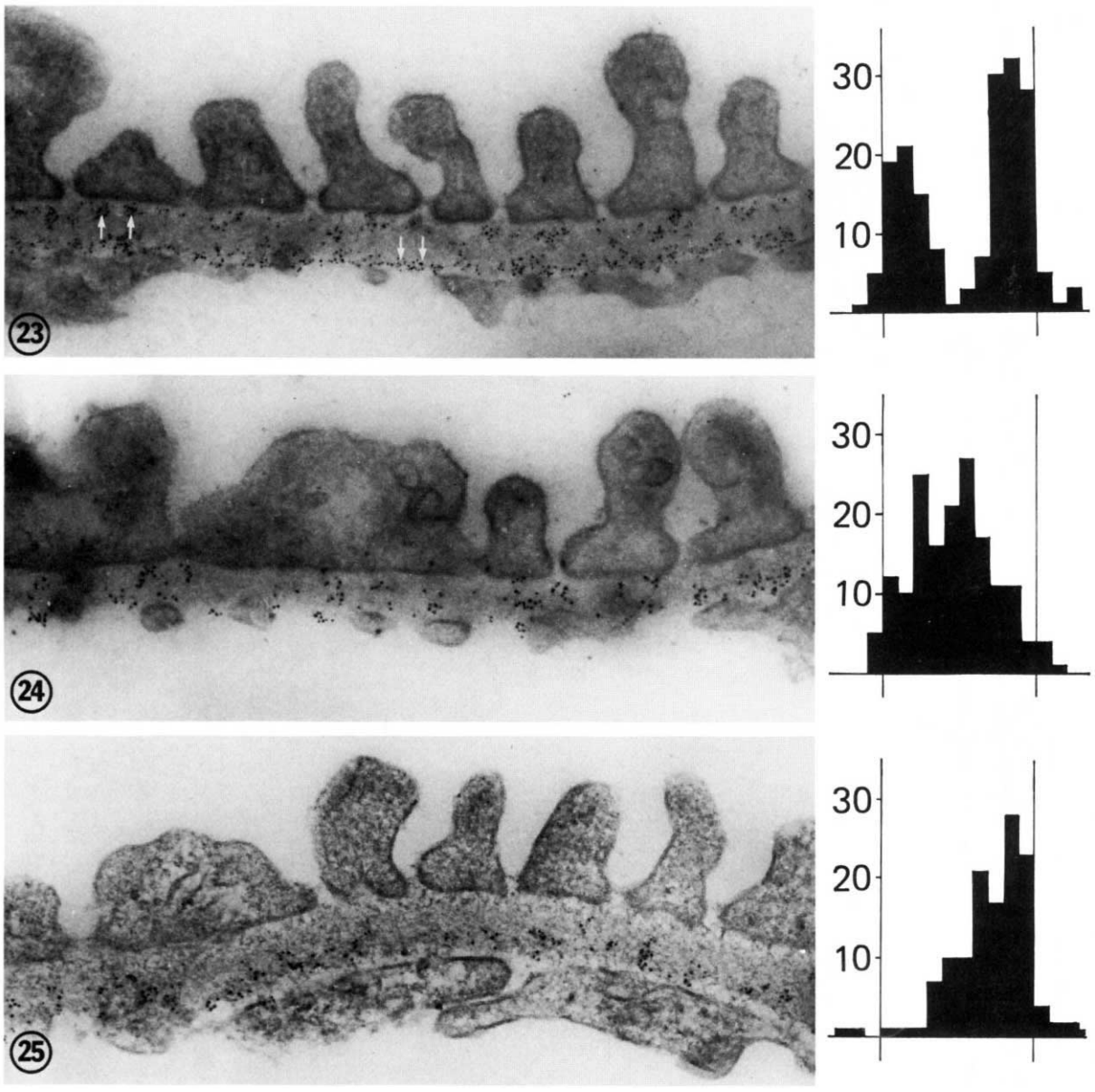
The use of GBM fractions for diffusion methods has the advantage that access of the antibody reagents to the GBM is facilitated by the removal of the overlying cell layers (endothelial, epithelial, mesangial). However, this approach, like the others, also has its limitations because some antigens could be lost during the isolation procedure, giving false negatives, or the structure of the basement membrane could collapse, thus limiting the resolution obtainable. Also, due to the limited permeability of the GBM, normally the large antibody probes would be expected to have access primarily to the surfaces of the isolated GBM. Notwithstanding these limitations, we have found immunolocalizations on isolated GBM to be useful in certain instances in diffusion methods with ferritin or gold conjugates for the localization of GBM-associated antigens. Laminin, fibronectin, and type IV collagen were localized to the GBM by an immunoferritin method [10, 84]. In addition, gp330, as well as anti-gp330 have been simultaneously localized by an immunogold method (using two different sizes of gold particles) in immune deposits in GBM fractions isolated from animals with HN [95] (Fig. 19). We have found the immunoperoxidase technique to be less useful for localizations on GBM fractions, because in isolated GBMs the reaction product is apparently at least partially lost by diffusion. This particular problem was demonstrated by the finding that, whereas both the antigen, gp330, and the antibody (anti-gp330) are regularly detected in immune deposits by immunoperoxidase methods in kidney tissue in situ and by immunogold methods in isolated GBM, only a few of the immune deposits appear reactive for either the antigen or antibody in isolated GBM fractions incubated by immunoperoxidase procedures.

Results obtained by i.v. injection of primary antibody.

In a few cases, antibodies have been introduced into the circulation of the living animal by i.v. injection, and the localization of the bound IgG was determined by a diffusion method, usually direct immunoperoxidase. This approach has proved to be valuable in obtaining information on the localization and accessibility to circulating antibodies of several GBM components, that is, heparan sulfate proteoglycans [9, 47] and laminin [46, 81]. When antibodies which specifically recognize the core proteins of basement membrane heparan sulfate proteoglycans (HSPG) were injected, they were found to bind



Figs. 20–22. *Examples of the diffusion and relocalization of DAB reaction product that can occur in the GBM in immunoperoxidase preparations.* Rats were injected intravenously with cationic ferritin (pI 7.4) to implant the protein in the laminae rarae [93, 94]. After 30 min the kidneys were fixed by perfusion (~1 min) with 3% glutaraldehyde–1.5% formaldehyde in 100 nm cacodylate buffer, pH 7.4. Cryostat sections were immunolabeled by indirect immunoperoxidase with either control IgG (Fig. 20) or rabbit-anti-ferritin Fab or IgG (Figs. 21–22). **Fig. 20.** In the control (incubated with nonimmune IgG), no DAB staining is observed. Ferritin molecules, visible by virtue of their dense iron cores, are largely restricted to the lamina rara inerna (LRI) and externa (LRE) of the GBM where they are found in discrete, regularly distributed (~60 nm) clusters. Very few ferritin molecules are seen in the lamina densa (LD) or along the plasmalemmae of endothelial (En) or epithelial (Ep) cells. **Fig. 21.** Restricted immunostaining pattern observed in a section incubated for 4 hr in 10 μg/ml of anti-ferritin Fab, and reacted briefly (2 min) in DAB. Reaction product is concentrated in the lamina rara interna and externa of the GBM where it is distributed at regular intervals (indicated by arrows), and is largely restricted to the ferritin clusters. Even under these more limited reaction conditions, some staining is also seen along the luminal surface of the endothelial plasmalemma (En) and at the base of the epithelial foot processes (fp), suggesting that some of the reaction product generated has diffused and bound to the adjoining cell membranes. **Fig. 22.** Extensive immunolabeling pattern observed in a section incubated for 4 hr in 10 μg/ml anti-ferritin IgG and reacted for 10 min in DAB. Under these more extended incubation conditions, reaction product has diffused and is distributed throughout the whole GBM and is also found along the entire endothelial plasmalemma (En) and the epithelial plasmalemma at the base of the foot processes (fp). The dense reaction product largely obscures discrete ferritin clusters in the laminae rarae. (Figs. 20–22, × 31,000.) Reproduced with permission from [28].



Figs. 23–25. *Immunogold labeling of the GBM in ultrathin frozen sections.* Pieces of rat kidney were fixed by perfusion with PLP, frozen in liquid N_2, and ultrathin sections were cut on a Reichert Ultracut Ultramicrotome equipped with a cryocutting attachment. The sections were mounted on electron microscope grids and incubated with a specific antibody followed by goat anti-rabbit IgG coupled to 10 nm colloidal gold. The number of gold particles in the layers of the GBM are expressed in the histograms accompanying each of the immunolocalizations. In **Fig. 23**, the section was incubated with anti-HSPG (GBM), an antibody that recognizes the core protein of basement membrane heparan sulfate proteoglycans [9]. Gold label is concentrated in both laminae rarae (arrows). In **Fig. 24**, from a section incubated with anti-laminin antibodies, gold particles are most concentrated in the lamina densa, whereas in **Fig. 25**, from a section incubated with anti-type IV collagen, most of the gold particles are localized on the inner half of the GBM. The right and left vertical lines on the histogram represent the positions of the endothelial and epithelial cell membranes, respectively, and the numbers indicate the number of gold particles. (Figs. 23–25, × 33,000.)

rapidly (within three minutes) only to the GBM where they were concentrated in the laminae rarae [9, 47]. The rapid and specific binding to the laminae rarae demonstrated that: 1) the specific population of basement membrane HSPG (Mr = 130,000) recognized by the antibodies are directly exposed to the circulation; 2) they are concentrated in the laminae rarae, which is in agreement with the findings obtained by surface methods [9] (Fig. 23), and 3) there is no cross-reactivity between the membrane-intercalated HSPG found on endothelial cell surfaces [96] and those found in the GBM, since there was no antibody binding to the endothelium. Interestingly, when the animals were followed over the next three to six months, antibody binding was found to induce a mild anti-GBM-type of glomerular disease and subepithelial thickening of the GBM in the recipient animals [47].

Using the same approach, laminin has been localized to the GBM. When HRP-labeled anti-laminin was administered i.v., it was detected throughout the GBM which was interpreted as

Table 1. Localization of basement membrane components in the GBM

Method	Distribution within the GBM	Source of immunogen	Pre-treatment	1st antibody	2nd antibody conjugate	Reference
Type IV collagen						
Diffusion	Lamina densa	EHS-sarcoma	None	Affi. IgG	HRP-Fab	[10]
Diffusion	Lamina densa with extensions into both laminae rarae	EHS-sarcoma	Sodium borohydride	Serum	Anti-rabbit IgG + PAP	[76]
i.v. injection	Lamina densa and laminae rarae	EHS-sarcoma	Sodium borohydride	Affi. IgG	Anti-rabbit IgG + PAP	[77]
Cryosection	Lamina densa	EHS-sarcoma	None	Affi. IgG	Biotin anti-rabbit IgG + avidin-ferritin	[78]
Cryosection	Inner half of lamina densa	EHS-sarcoma	None	Affi. IgG	Anti-rabbit–IgG-gold	This paper
Lowicryl section	Lamina densa	EHS-sarcoma	None	Serum	Anti-rabbit–IgG-gold	[79]
Laminin						
Diffusion	Laminae rarae	EHS-sarcoma	None	Affi. IgG	Anti-rabbit Fab-HRP	[10]
Diffusion	Lamina densa with extensions into both laminae rarae	EHS-sarcoma	Sodium borohydride	IgG	Anti-rabbit IgG + PAP	[76]
Diffusion	Preferentially in the Laminae rarae	EDS-PYS	Sodium borohydride	Affi. IgG	Anti-rabbit IgG + PAP	[80]
i.v. injection	More intense in laminae rarae	EHS-sarcoma	Sodium borohydride	Affi. IgG	Anti-rabbit IgG + PAP	[77]
i.v. injection	Lamina densa and laminae rarae	EHS-sarcoma	None	HRP-Fab	None	[81]
i.v. injection	Lamina densa and laminae rarae	EHS-sarcoma	None	HRP-IgG	None	[46]
Cryosection	Lamina rara interna	EHS-sarcoma	None	Affi. IgG	Biotin-anti-rabbit IgG + avidin-ferritin	[82]
Cryosection	Mainly lamina densa	EHS-sarcoma	None	Affi. IgG	Anti-rabbit IgG-gold	This paper
Heparan sulfate proteoglycans						
Diffusion	Lamina densa and extensions into both laminae rarae	EHS-sarcoma	Sodium borohydride	IgG	Anti-rabbit IgG + PAP	[76]
Diffusion	Variable results	Rat glomeruli	None	IgG	Anti-rabbit Fab-HRP	[89]
i.v. injection	More intense in both laminae rarae	EHS-sarcoma	Sodium borohydride	Antibody	Anti-rabbit IgG + PAP	[77]
i.v. injection	Both laminae rarae	Rat glomeruli	None	IgG	Anti-rabbit Fab-HRP	[9, 47]
Cryosection	Both laminae rarae	Rat glomeruli	None	IgG	Anti-rabbit IgG-gold	[9]

indicating the presence of laminin in all layers of the GBM [46, 81]. This is in agreement with some studies, but not with others (Table 1). Anti-laminin binding was also shown to induce thickening of the GBM [46].

Results obtained by surface methods

Surface methods have also been utilized extensively for localization of GBM antigens. They have the advantage, already alluded to, that the problem of accessibility of antigens as well as that of DAB diffusion are avoided since they are carried out at the section level with ferritin or gold conjugates. They have the disadvantage, however, that the sensitivity of the method is low. We have recently localized several GBM antigens on both ultrathin frozen sections and on sections cut from tissues embedded in Lowicryl K4M. The most definitive results were obtained with the localization of basement membrane HSPG [9]. Using the antibody to the core protein of the Mr = 130,000 HSPG mentioned above, we localized these HSPG to the laminae rarae of the GBM by an immunogold procedure in both ultrathin frozen [9] (Fig. 23) and Lowicryl sections. These results are in accord with the results of direct immunoperoxidase staining after in vivo antibody injections [9]. Indirect immunoperoxidase localizations gave more variable results [9]; however, because convergent results were obtained by three different approaches—immunogold staining on frozen thin sections, immunogold staining on Lowicryl K4M sections and direct immunoperoxidase staining—we concluded with some confidence that these HSPG are concentrated in the laminae rarae of the GBM [9].

When laminin and type IV collagen were similarly localized on ultrathin sections the results were less satisfying. To begin with, we were unable to detect either antigen in Lowicryl K4M sections using immunogold reagents. This is in keeping with our experience that a number of antigens cannot be detected by this procedure that can be detected by other methods.

Localizations using immunogold reagents on ultrathin frozen sections were more consistently successful (Figs. 24–25). We found laminin to be localized all across the basement membrane, but it was most concentrated (based on counts of gold particles) in the lamina densa, whereas type IV collagen was localized mostly on the inner half of the lamina densa. These results differ from many previous reports, including our own based on indirect immunoperoxidase localizations [10] using the same primary antibodies. They are even different from the results obtained with surface methods reported from other laboratories (Table 1). To avoid the possibility that some of the antigenic sites of these proteins are masked, sections were digested with enzymes (collagenase, proteases) or treated with reducing agents (Na borohydride), but no differences were observed in the overall localizations in treated and untreated

sections. Therefore, it is very difficult at present to explain the discrepancies in the localization of antigens in the various layers of the GBM obtained by different immunocytochemical techniques.

Several researchers have proposed that type IV collagen is concentrated in, and is the major component of the lamina densa, whereas laminin is concentrated in the laminae rarae [97], where it serves to attach the plasma membranes of the epithelial and endothelial cells to the type IV collagen in the lamina densa [98]. However, in view of the discrepant results obtained, it may be wise to limit one's observations to recording the presence or absence of a given antigen in the GBM, and to postpone drawing conclusions concerning the presence or absence of any particular antigen in the individual layers of the GBM until we better understand the vagaries of individual immunocytochemical methods and unless there is convergent information available from more than one immunocytochemical or alternative technique.

Conclusions and outlook

We have presented here a summary of our experiences which indicate that immunoelectron microscopy can be used as a major analytical tool in kidney research. Recent developments which have provided improvements and simplifications of existing methods make it possible to obtain good structural preservation as well as accurate localization of antigens in cell organelles and extracellular matrix components. Thus, immunocytochemistry not only provides a valuable supplement to biochemical and immunological analyses, but also it frequently provides information that is difficult or impossible to obtain by other procedures. Frequently immunocytochemical results have provided unexpected findings (such as, the localization of the Heymann nephritis antigen in coated pits) that have directed the next step in our experimental and intellectual approach to the cellular and molecular analysis of various renal problems, such as the pathogenesis of membranous nephropathy and minimal change nephrosis.

We have also presented here some of the difficulties that we have encountered in carrying out immunolabeling at the electron microsope level, as exemplified by attempts to localize accurately antigens within the different layers of the GBM. These difficulties serve to emphasize the need to be rigorous and cautious in interpreting data, to be aware of the limitations as well as the advantages of these methods, and to seek convergent information obtained from more than one approach wherever possible.

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